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14. ABSTRACT:

Clinical data indicate a link of disease progression and resistance to imatinib therapy.While previous work on imatininb resistance has focused on mutations in the drug target,these cannot explain the reduced effectiveness of imatinib in advanced disease stages. Usinga candidate approach we found that the p53 tumor suppressor is activated by and contributesto the antitumor activity of imatinib. Further, p53 is implicated in disease progression inCML (Wendel, et al. Proc Natl Acad Sci U S A. 2006 May 9;103(19):7444-9).To further approach the problem using an unbiased approach, we proposed an in vivo RNAiscreen. This turned out to be technically extremely challenging and we had to take a verysystematic approach at resolvng some of the problems. However, we have developed i) animproved RNAi design, ii) suiotable library vectors for in vivo and in vitro work, iii)nearly genome wide libraries in these vectors, and iv) improved, array-based detectionmethods. With these necessary and important improvements we now have promising preliminarydata from in vitro screens.

15. SUBJECT TERMS
CML progression, imatinib resistance, RNAi screen, p53, tumor suppression

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INTRODUCTION:

The purpose of the proposed work is to identify genetic changes involved in disease progression in CML and analyze if they impact sensitivity to imatininb therapy. Our approach is using a relevant mouse model of CML and *in vivo* RNAi technology. We have been able to characterize the role of the *p53* tumor suppressor gene in progression and imatininb response (1). The in vivo RNAi screening is technologically very challenging and we took a very methodic approach to improving this technology. However this aspect of the work is ongoing.

The original term of the grant was 12 months, however, in 2005 I was granted a no-cost, one-year extension. This has allowed me to follow an important lead on *p53* in CML, which has resulted in a publication in the Proc. of the Nat'l. Acad. of Science (1)(attached). We have also been able to improve the shRNA design, the retroviral vectors and methods for RNAi detection and conduct *in vitro* RNAi screens for drug resistance in tumor cells.

Personally, I was appointed to the Cancer Biology and Genetics program at Sloan-Kettering and will set up my laboratory in January 2007. Notably, work sponsored by this grant – and in particular the ongoing collaborations with the Lowe and Hannon labs in Cold Spring Harbor - contributed in a major way to my training as an independent researcher.

BODY:

The biology of **disease progression in CML** is incompletely understood. Several genetic lesions have been linked with progression, including loss of tumor suppressors *p16, Rb* or *p53*. Importantly, disease progression is often associated with a reduced effectiveness of imatinib therapy. However, in the chronic phase of CML most cases of resistance can be explained by *Bcr-Abl* point mutations (2), the increase in drug resistance upon progression can not be explained by these mutations, in fact in some studies refractory cases in advanced disease stages show a *lower* frequency of *Bcr-Abl* mutations (3). These clinical data suggest, that factors associated with or directly involved in disease progression may impact imatinib therapy. Our work addresses the role of genetic changes in disease progression and explores their impact on imatinib response – ultimately this might open new therapeutic opportunities.

We proposed to conduct an **in vivo RNAi screen in the Tec p210 transgenic mouse**, a long latency model of chronic myeloid leukemia (4), to identify genetic lesions that would cause acceleration and a more aggressive pathology. In a second study, we would then study the impact of these lesions on imatinib response. Using a highly innovative technology, we put great emphasis on accurate positive and negative controls. Loss of *p53* has been shown to cause disease acceleration in the Tecp210 CML model (5).

We initiated experiments to confirm the **effect of *p53* loss in a retroviral model of CML** (6). Basically, we transduced hematopoietic stem cells (HSCs) of different *p53* status with a Bcr-Abl expressing retroviral vector **(see Appendix: Wendel et al., Fig. 2A)**. Briefly, we found some acceleration of disease onset and variable effects on the pathological appearance **(see**

Appendix: Wendel et al., Figs. 2B and 2C). However, in a *p53* heterozygous background we consistently observed loss of heterozygosity (LOH) indicating selection for *p53* loss in this *Bcr-Abl* driven disease **(Appendix: Wendel et al., Fig 2C, inset)**. Most strikingly, however, we found that while *p53*+/+ CML bearing mice showed complete responses under imatininb therapy and even a positive effect on survival, mice bearing *p53* deficient leukemias showed only partial responses and rapidly succumbed **Appendix: Wendel et al., Fig. 3C and Suppl. Fig. 6)**. This reduced imatinib effect is important, because inactivation of *p53* has been reported in up to 1/3 of patients with advanced disease – thus, while not identified in the proposed screen *p53* appeared to be exactly what we were looking for and we decided to follow this lead.

As **resistance to imatininb** had mostly been attributed to impaired drug-target interaction and consequently reduced kinase inhibition, we first wanted to ascertain equal BCR-ABL inhibition by imatininb in mice with *p53*+/+ and *p53*-/- leukemias. Looking at both direct and indirect targets of BCR-ABL signaling, p62Dok and ribosomal S6 protein, respectively, we found equal effect of the drug on kinase activity (**Appendix: Wendel et al., Fig. 3D)**. Indicating, that lack of response was due to a post-target mechanism.

Next, we wanted to **validate our *in vivo* findings using an established *in vitro* system** of *Bcr-Abl* mediated transformation. We chose 32D and Baf/3 cell lines, but also looked at colony formation by primary HSCs transduced with *Bcr-Abl*. Briefly, 32D and Baf/3 cells were transduced with *Bcr-Abl* and selected by IL-3 withdrawal. Subsequently we introduced either of two RNAi vectors effectively knocking down *p53* and control vector into a fraction of the *Bcr-Abl* expressing cells. The RNAi vectors co-expressed GFP and thus we could use simple '*in vitro*

competition experiments' to test for enrichment of the *p53* RNAi expressing cells under imatinib treatment. Notably, both RNA vectors conferred an advantage in *Bcr-Abl* expressing cells under imatinib treatment and were massively enriched, this effect was not observed in cells expressing an imatinib insensitive mutant of *Bcr-Abl* (T315I) (**Appendix: Wendel et al., Fig. 1D and Suppl. Fig. 2)**. Along with colony formation data **(Fig. 2C)**, these findings indicate that the tumor suppressor *p53* contributes to imatinib action at least in part through a cell-intrinsic effect and resistance *in vivo* is not merely a reflection of a more aggressive pathology.

Then we wanted to understand the **effect of BCR-ABL inhibition on *p53***. We found that imatinib (and similarly other Bcr-Abl inhibitors) activate the tumor suppressor *p53* in most cell lines (Baf/32d and HSC) and also *in vivo*. This effect was restricted to cells expressing *Bcr-Abl* and was not observed in either parental cells or cells expressing the T315I mutant of *Bcr-Abl* – thus ruling out off-target effects of the pharmacologic inhibitors (**Appendix: Wendel et al., Fig. 1A and Fig. 4A)**. To address a possible **mechanism** for *p53* induction upon BCR-ABL inhibition, we analyzed the effect of imatinib treatment on BCR-ABL downstream effectors. Strikingly, Akt mediated activation of Mdm2 as mediated by a S166 phosphorylation was sharply reduced upon imatinib treatment *in vitro*. A direct effect on Mdm2 levels was not observed (7) (**Appendix: Wendel et al., Fig. 1B)**. Clearly, the interaction between *Bcr-Abl* and *p53* is more complex, however, these findings suggest a mechanism for the observed *p53* activation as a consequence of inhibiting signals emanating from the BCR-ABL kinase.

Finally, we analyzed matched **samples from CML patients**, for the occurrence of *Bcr-Abl* and *p53* mutations in collaboration with the group of A. Hochhaus in Heidelberg, Germany.

We selected patients who were in various disease stages and for whom matched samples, before and after the onset of resistance, were available and who did not have detectable *p53* mutations at the start of imatinib therapy. Indeed, upon imatinib resistance we found in 3/5 patients the occurrence of novel *p53* mutations and absence of *Bcr-Abl* mutations; 2 of these *p53* mutations were at typical 'hotspot' sites **Appendix: Wendel et al., Suppl. Table 1)**. While only a small set, these data indicate that loss of *p53* can accompany the onset of imatinib resistance in patients.

In **conclusion**, we reported that *p53*, a tumor suppressor that has been implicated in disease progression in CML, contributes to the antitumor activity of imatinib, such that loss of *p53* may account, in part for the increase in resistance seen in later disease stages. This study is important because it illustrates that a non-target mutation can mediate resistance to a targeted therapeutic (1). In fact, our study has sparked similar analyses in solid tumors resistant to EGFR inhibitors. Ultimately, understanding the determinants of response to targeted therapeutics should lead to improved therapies, e.g. combinations.

Completing and publishing the data outlined above has been a priority for me and has to some extent delayed **progress on the RNAi screen**. However, we have made significant and rather methodical progress towards developing RNAi technology for *in vivo* screens.

The library we originally proposed to use contained 6 vectors against 1000 genes (the 'Cancer 1000' collection), the hairpins in this library were RNA stem loops of 19-29 nucleotides which were expressed from the U6 promoter. It has become clear, that the RNAi context is

extremely important for function, for example naturally occurring micro RNAs have extensive flanking regions. Based on these findings, we have transferred at first individual vectors into this **micro RNA context**, specifically the mir30 context, and indeed found far superior knockdown of the targeted gene expression (8). Subsequently, as reported in 10/2005 we have transferred the 'Cancer 1000' collection into this context and since then have further expanded the collection and now have almost complete coverage of the mouse and human genomes in the micro RNA context.

Next, we improved the **vector design**. We found that the U6 (Pol III promoter) was inferior to Pol II promoters for expression of the RNAi in the micro RNA context and thus moved from the self-inactivating (SIN) MSCV vector to an MSCV vector containing intact 3' and 5' LTRs directing expression of the RNAi. Also the puromycin cassette posed a problem in *in vivo* studies – likely due to an immune response. Thus we have developed separate MSCV based vectors for *in vitro* and *in vivo* experiments, the first contains the PuroR the latter expresses GFP from an SV40 promoter instead of the PuroR cassette.

Using this vector design we have now conducted some ***in vitro* screens** for resistance to chemotherapeutic drugs in lymphoma cells and preliminary data look promising. This has also served to improve the detection technology. Initially, we proposed to use PCR and subsequent sequence analysis to detect enrichment of individual RNAi's. Recently, we had some success using an array-based approach, where a half-hairpin is printed on the chip. Genomic DNA from tumor cells is isolated and the RNAi PCR amplified and subsequently hybridized. This approach

should facilitate positive screens and might even enable negative screens for RNAi vectors that are lost from the population.

We are currently conducting ***in vivo*** **experiments** using the *p53* RNAi vector as a positive control to reconstruct the library. The purpose of the reconstruction is to define the maximum pool size that will allow identification of a scoring RNAi. These experiments are ongoing and critical as a positive control and to determine pool size.

Overall, the technology for in *vivo RNAi* screens has presented us with several challenges as outlined above and we have taken a very systematic approach at addressing these problems. Notably despite tremendous interest, to date no other lab has been able to report an *in vivo* RNAi screen. We have made significant progress and continue to develop the technology in order to achieve the first *in vivo* RNAi screen.

KEY RESEARCH ACCOMPLISHMENTS:

- The *p53* tumor suppressor gene contributes to the antitumor activity of imatinib *in vivo* and is implicated in disease progression
    - Imatinib activates the *p53* tumor suppressor
    - A non-target mutation can modulate response to a targeted drug
    - *P53* contributes to tumor cell 'addiction' to oncogenic signals
    - Finding may explain the increase in imatininb resistance in advanced disease
    - *P53* mutations occur in patients upon imatinib resistance

- Technology development for RNAi library screen
    - RNAi design using natural mir30 context
    - Design of retro- and lentiviral vectors for *in vitro* and *in vivo* expression of RNAi
    - Establishment of detection technology using an array-based platform
    - Expanded (~genome wide) mouse RNAi library in mir30 context in both retro- and lenti-viral vectors

REPORTABLE OUTCOMES:

- Publication: **Hans-Guido Wendel**, Elisa de Stanchina, Enrique Cepero, Sagarika Ray, Jordan S. Friedman, Michael Emig, Darren R. Veach, William G. Bornmann, Bayard Clarkson, W. Richard Mc Combie, Andreas Hochhaus, Scott C. Kogan, and Scott W. Lowe. p53 and targeted cancer therapy. **Proc Natl Acad Sci U S A** 2006;103:7444-9.
- Numerous oral and poster presentations at national and international meetings
- Patent application: P53 Determines In Vivo Response To Targeted Therapy in Human CML (US provisional patent application)
- Adaptation of Warren Pear's retroviral CML model to study impact of secondary genetic lesions on pathology and treatment response
- Collaboration with Lowe and Hannon labs at Cold Spring Harbor on RNAi vector and library development
- Improved RNAi design using mir30 context
- Design of retro- and lenti-viral vectors for effective in vitro and in vivo RNAi expression
- Establishment of array based RNAi detection platform
- Critical step in my scientific career as I was appointed as Assistant Member in the Cancer Biology & Genetics Dept. at Sloan-Kettering Institute

CONCLUSION:

We set out to conduct an RNAi screen to identify genetic lesions that might explain the observed increase in imatinib resistance upon progression in CML. The RNAi screen presented us with major technical challenges, which we addressed in a very systematic fashion. In a candidate approach, however, we found that loss of *p53*, which has been implicated in disease progression in CML, also conferred resistance to imatinib therapy. This finding is surprising, because to date resistance to targeted therapy was almost exclusively attributed to mutations in the direct drug target. We show that a common, non-target mutation can impede targeted therapy. Based on this, other groups are currently looking at the role of *p53* loss in solid tumors, e.g. lung cancer, refractory to EGFR inhibitor therapy. Our findings have thus broadened our understanding of the factors determining response to targeted therapy and will hopefully lead to new therapeutic opportunities.

With respect to the RNAi screening technology, we have accomplished important improvements in vector and RNAi design, which come to benefit us and others in our continued effort to use this technology for *in vivo* screens.

Finally, the collaboration with the Lowe and Hannon labs, in particular in overcoming some of the obstacles in the RNAi project, has been a valuable training experience and presentation and discussion of the work accomplished with the Army sponsorship has enabled me to attain an independent position at Sloan-Kettering.

REFERENCES:

1. Wendel, H. G., de Stanchina, E., Cepero, E., Ray, S., Emig, M., Fridman, J. S., Veach, D. R., Bornmann, W. G., Clarkson, B., McCombie, W. R., Kogan, S. C., Hochhaus, A., and Lowe, S. W. Loss of p53 impedes the antileukemic response to BCR-ABL inhibition. Proc Natl Acad Sci U S A 2006;103:7444-9.
2. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001;293:876-80.
3. Lahaye, T., Riehm, B., Berger, U., Paschka, P., Muller, M. C., Kreil, S., Merx, K., Schwindel, U., Schoch, C., Hehlmann, R., and Hochhaus, A. Response and resistance in 300 patients with BCR-ABL-positive leukemias treated with imatinib in a single center: a 4.5-year follow-up. Cancer 2005;103:1659-69.
4. Honda, H., Oda, H., Suzuki, T., Takahashi, T., Witte, O. N., Ozawa, K., Ishikawa, T., Yazaki, Y., and Hirai, H. Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210bcr/abl: a novel transgenic model for human Ph1-positive leukemias. Blood 1998;91:2067-75.
5. Honda, H., Ushijima, T., Wakazono, K., Oda, H., Tanaka, Y., Aizawa, S., Ishikawa, T., Yazaki, Y., and Hirai, H. Acquired loss of p53 induces blastic transformation in p210(bcr/abl)-expressing hematopoietic cells: a transgenic study for blast crisis of human CML. Blood 2000;95:1144-50.
6. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L., and Baltimore, D. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood 1998;92:3780-92.
7. Trotta, R., Vignudelli, T., Candini, O., Intine, R. V., Pecorari, L., Guerzoni, C., Santilli, G., Byrom, M. W., Goldoni, S., Ford, L. P., Caligiuri, M. A., Maraia, R. J., Perrotti, D., and Calabretta, B. BCR/ABL activates mdm2 mRNA translation via the La antigen. Cancer Cell 2003;3:145-60.
8. Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Hannon, G. J., and Lowe, S. W. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet 2005;37:1289-95.

Personel receiving pay from the research effort:

Hans-Guido Wendel

List of Publications

Wendel, H. G., de Stanchina, E., Cepero, E., Ray, S., Emig, M., Fridman, J. S., Veach, D. R., Bornmann, W. G., Clarkson, B., McCombie, W. R., Kogan, S. C., Hochhaus, A., and Lowe, S. W. Loss of p53 impedes the antileukemic response to BCR-ABL inhibition. **Proc Natl Acad Sci U S A** 2006;103:7444-9.

Selected meetings at which the work has been presented either as a Talk or Poster presentation:

70th Cold Spring Harbor Symposium Molecular Approaches to Controlling Cancer, Cold Spring, June 1 - 6, 2005 Harbor, 2005

CSHL Meeting on Mechanisms & Models of Cancer, Cold Spring Harbor, August 16-20, 2006

ESH/AACR Conference on Molecular Basis for Targeted Therapy for Leukaemia Cascais, Portugal, 2-5 February, 2006

APPENDIX:

1) Publication:

Wendel, H. G., de Stanchina, E., Cepero, E., Ray, S., Emig, M., Fridman, J. S., Veach, D. R., Bornmann, W. G., Clarkson, B., McCombie, W. R., Kogan, S. C., Hochhaus, A., and Lowe, S. W. Loss of p53 impedes the antileukemic response to BCR-ABL inhibition. **Proc Natl Acad Sci U S A** 2006;103:7444-9.

2) Supplementary Information:

- Legends for Supplementary Figures
- Supplementary Figures 1-6
- Supplementary Table 1

Loss of *p53* impedes the antileukemic response to BCR-ABL inhibition

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Targeted cancer therapies exploit the continued dependence of cancer cells on oncogenic mutations. Such agents can have remarkable activity against some cancers, although antitumor responses are often heterogeneous, and resistance remains a clinical problem. To gain insight into factors that influence the action of a prototypical targeted drug, we studied the action of imatinib (STI-571, Gleevec) against murine cells and leukemias expressing BCR-ABL, an imatinib target and the initiating oncogene for human chronic myelogenous leukemia (CML). We show that the tumor suppressor p53 is selectively activated by imatinib in BCR-ABL-expressing cells as a result of BCR-ABL kinase inhibition. Inactivation of p53, which can accompany disease progression in human CML, impedes the response to imatinib *in vitro* and *in vivo* without preventing BCR-ABL kinase inhibition. Concordantly, p53 mutations are associated with progression to imatinib resistance in some human CMLs. Our results identify p53 as a determinant of the response to oncogene inhibition and suggest one way in which resistance to targeted therapy can emerge during the course of tumor evolution.

imatinib | mouse model | targeted therapy | drug resistance | tumor-suppressor gene

Most conventional cancer therapies were identified through empirical screens for agents that preferentially kill tumor cells compared with normal tissues. Although these agents are effective in treating some human cancers, many tumors are nonresponsive or evolve to a resistant state. More recently, insights into the molecular basis of cancer have enabled the development of more rational drugs that attack activities involved in the oncogenic process (1). These "targeted" therapeutics are often less toxic than conventional drugs and have remarkable activity against some cancers. The effectiveness of such agents may reflect the cancer cell's continued dependency on an oncogenic lesion such that it cannot tolerate the absence of its signal, a phenomenon sometimes referred to as "oncogene addiction" (2, 3). However, the molecular determinants that contribute to the sensitivity and resistance of tumor cells to targeted therapies are poorly understood.

Imatinib (STI-571, Gleevec) is a small-molecule inhibitor of BCR-ABL whose effectiveness against CML makes it the paradigm for targeted cancer therapy. Clinical resistance to imatinib is most often caused by point mutations in the BCR-ABL kinase that prevent the drug–target interaction and subsequent kinase inhibition (4), an observation that provides formal proof that the BCR-ABL kinase is essential for imatinib sensitivity and that has produced rational strategies to circumvent drug resistance (5, 6). However, imatinib is generally less effective against Ph^+ (Philadelphia chromosome) acute lymphoblastic leukemia or CML that has progressed to a more genetically complex phase known as "blast crisis," a response pattern that cannot be entirely explained by the appearance of BCR-ABL mutants (7–9). Presumably, other factors associated with disease progression can impede imatinib action.

The *p53* tumor suppressor provides a potent barrier to tumorigenesis by triggering cell-cycle checkpoints, cellular senescence, or apoptosis in response to DNA damage and aberrant proliferative signals (10, 11). Because conventional chemotherapeutic agents can exploit the same signaling networks influenced by *p53* for their optimal antitumor effects, *p53* mutations acquired during tumorigenesis can promote drug resistance as a byproduct of tumor evolution (12). However, how *p53* influences the response of tumors to targeted cancer therapy is not known. Interestingly, *p53* mutations can accompany disease progression in human CML (13–17), and there is substantial cross-talk between the *BCR-ABL* and *p53* signaling networks (18–22). Here, we examine the impact of *p53* on the cellular response to imatinib in several well characterized models of *BCR-ABL*-induced malignant transformation. Our results illustrate how factors linked to malignant progression can modulate the response of tumor cells to targeted cancer therapy and have implications for understanding the heterogeneous responses to these therapies in the clinic.

Results

BCR-ABL Kinase Inhibition Induces *p53*. To examine the impact of *p53* on imatinib responses *in vitro*, we first examined Ba/F3 cells, a murine Pro-B cell line that has been used extensively to study *BCR-ABL* transformation and imatinib response (5). Cells were infected with retroviruses expressing a control vector, BCR-ABL (p210), or a BCR-ABL mutant that is insensitive to imatinib (p210/T315I), and the resulting populations were assessed for p53 levels and BCR-ABL kinase activity 8 h after imatinib addition.

As expected, p53 was expressed at low levels in the parental cells and was not induced after imatinib treatment. In contrast, upon imatinib treatment, p53 was induced in BCR-ABL (p210)-expressing cells but not those expressing the BCR-ABL mutant (T315I). Phosphorylation of $p56^{DOK\text{-}2}$, which is a downstream target of BCR-ABL signaling, was high in both BCR-ABL-expressing populations and was repressed in treated cells expressing p210 but not the p210/T315I mutant (Fig. 1*a*). Similar results were observed in 32D cells and after treatment with PD166326, a dual BCR-ABL/SRC kinase inhibitor with a distinct chemical structure (6) (Fig. 5, which is published as supporting information on the PNAS web site, and data not

Conflict of interest statement: No conflicts declared.

Freely available online through the PNAS open access option.

Abbreviations: CML, chronic myelogenous leukemia; HSC, hematopoietic stem cell; RNAi, RNA interference.

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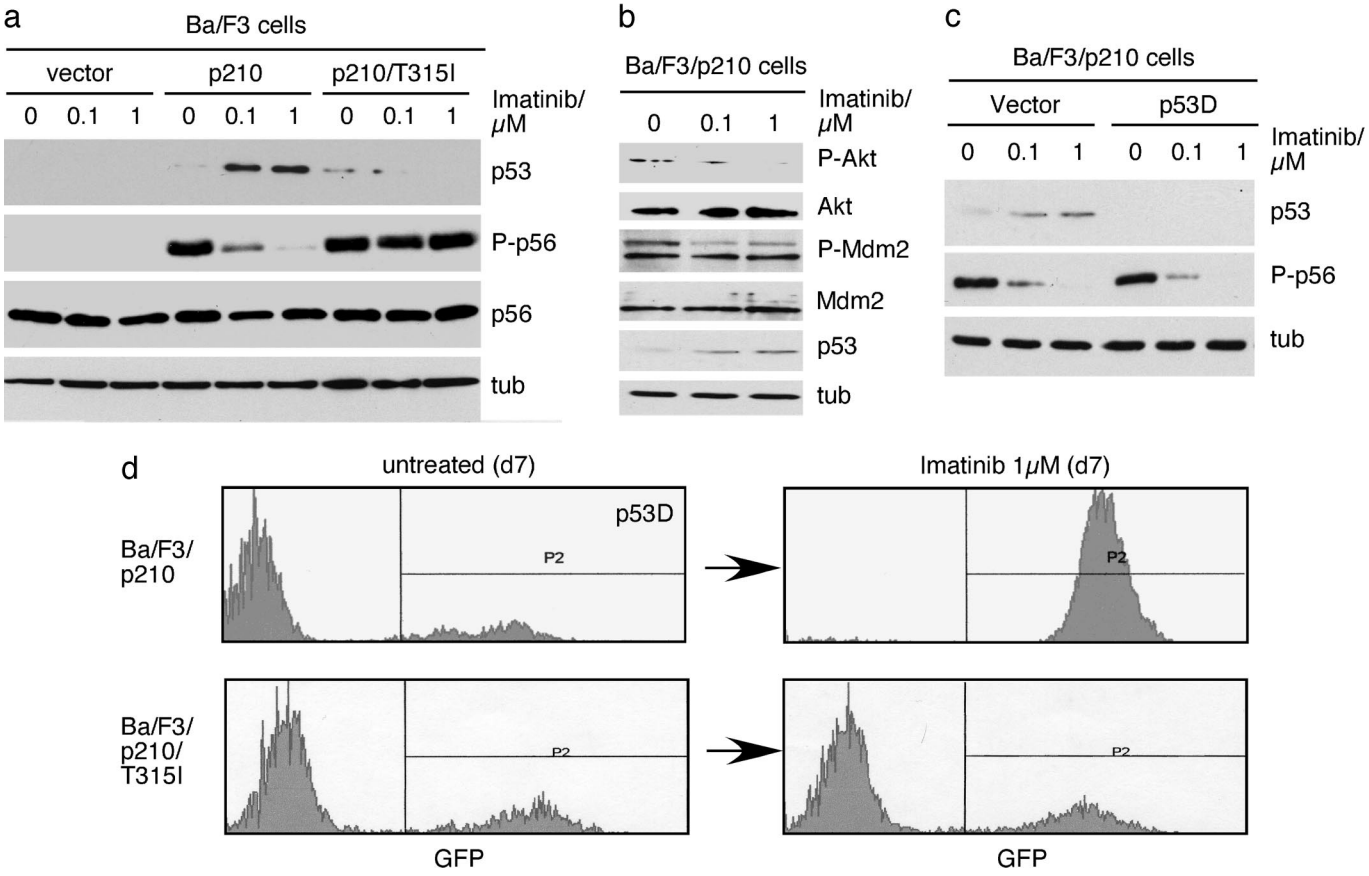

Fig. 1. *p53* modulates sensitivity to imatinib *in vitro*. (*a*) Immunoblot analysis of lysates prepared from Ba/F3 cells stably transduced with empty vector, BCR-ABL (p210), or mutant BCR-ABL (p210/T315I) treated with imatinib for 8 h as indicated were probed for p53, phosphorylated; and total levels of $p56^{Dok\text{-}2}$ protein (P-p56 and p56, respectively), and tubulin (tub) as loading control. (*b*) Lysates of imatinib-treated Ba/F3/p210 cells were probed with antibodies against p53, total and phosphorylated (Ser-473) Akt (Akt and P-Akt), and total and phosphorylated (Ser-166) Mdm2 (Mdm2 and P-Mdm2), with tub as loading control. (*c*) Immunoblot of Ba/F3/p210 cells lysates expressing either an RNAi vector targeting *p53* (p53D) or control vector (Vector) treated as indicated and probed for p53, phosphorylated (P-p56) and total (p56) p56 protein, and tub. (*d*) *In vitro* competition assay. Populations of Ba/F3 cells stably expressing either BCR-ABL (p210; *Upper*) or the T315I mutant (p210/T315I; *Lower*) were partially transduced with an RNAi vector against *p53* (p53D) and propagated in the presence or absence of 1 μM imatinib for 1 week and then subjected to flow cytometry to determine the fraction of cells containing the RNAi vector (high GFP expression).

shown). However, in a T cell leukemia line expressing BCR-ABL (BV173), significantly higher concentrations of kinase inhibitor were needed to induce p53 levels (e.g., 500 nM PD166326, data not shown), suggesting that cell-line-specific differences can exist (22).

BCR-ABL promotes oncogenesis through several downstream effectors, including the Akt/PKB kinase (23). Akt also phosphorylates Mdm2 on Ser-166, which acts to prevent Mdm2 from efficiently targeting p53 for degradation (24). Interestingly, phosphorylation of Akt/PKB was reduced in response to imatinib in p210-expressing BaF/3 cells, coinciding with a parallel decrease in Ser-166-phosphorylated Mdm2 (Fig. 1*b*). Together, these data imply that imatinib activates p53, at least in part, through inhibition of BCR-ABL signaling.

***p53* Contributes to Imatinib Action *in Vitro*.** To evaluate the impact of *p53* on imatinib action, we used RNA interference (RNAi) to suppress *p53* and determined how this RNAi impacted cellular responses to the drug. We introduced a retroviral vector coexpressing one of two different *p53* short hairpin (sh)RNAs [p53C (25) and p53D (26)] with GFP into Ba/F3 cells expressing p210 or p210/T315I, and the percentage of GFP positive cells was assessed by flow cytometry. FACS-purified GFP-positive cells expressing the *p53* shRNA showed substantial p53 knockdown and prevented p53 induction after imatinib treatment, although BCR-ABL kinase activity was inhibited as indicated by the reduction in phosphorylated $p56^{Dok\text{-}2}$ protein, a BCR-ABL target (Fig. 1*c*). Treatment of the mixed populations with 1 μM imatinib revealed that p53 knockdown with either shRNA conferred a selective advantage to Ba/F3 cells expressing p210, because the fraction of RNAi/GFP-expressing cells increased substantially (Fig. 1*d Upper*; and see Fig. 6, which is published as supporting information on the PNAS web site). By contrast, the RNAi vector targeting *p53* conferred no advantage to Ba/F3 cells expressing the imatinib-resistant p210/T315I mutant and neither did a control vector (Figs. 1*d Lower* and 6). Similar results were observed in BCR-ABL-expressing derivatives of the 32D myeloid cell line (see Fig. 7, which is published as supporting information on the PNAS web site). Thus, *p53* can contribute to the antileukemic effects of imatinib *in vitro*.

We next examined the relationship between *p53* and imatinib action in primary cultures enriched for hematopoietic stem cells (HSCs). To this end, we isolated and expanded fetal liver cells derived from crosses of $p53^{+/-}$ mice. We transduced these cells with a retroviral vector encoding BCR-ABL and measured p53 expression after imatinib treatment. Similar to our findings in the established Ba/F3 cell line, we found that *in vitro* treatment of $p53^{+/+}$-enriched HSCs caused decreased activation of Akt and Mdm2 and induction of p53 across a range of imatinib concentrations (Fig. 2*a*).

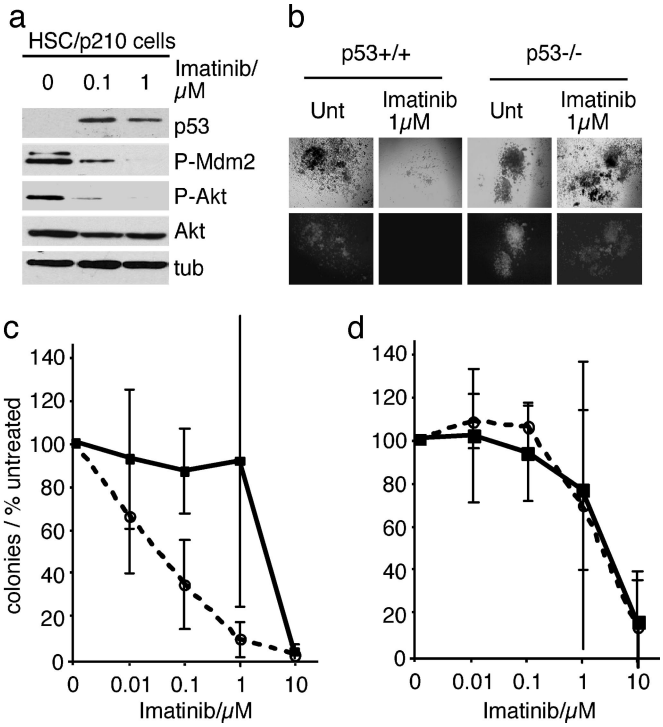

Fig. 2. BCR-ABL sensitization of primary HSCs to imatinib depends on *p53*. (*a*) Immunoblot analysis of $p53^{+/+}$ HSCs expressing BCR-ABL before therapy (Untr) or 8 h after treatment with different doses of imatinib, as indicated. Lysates were immunoblotted for p53, total and phosphorylated (Ser-473) Akt (Akt and P-Akt), phosphorylated (Ser-166) Mdm2 (P-Mdm2), and tubulin (tub). (*b*) Representative microphotographs of colonies formed by $p53^{+/+}$ or $p53^{-/-}$ HSCs in methylcellulose untreated or treated with 1 μM imatinib (*Lower*) fluorescence detection of GFP expression in *BCR-ABL*-transformed colonies. (*c* and *d*) Results of methylcellulose colony-formation assays, $p53^{+/+}$ (circles) and $p53^{-/-}$ (squares) HSC expressing BCR-ABL (*c*) or control (*d*) were incubated with imatinib at the indicated concentrations and colony-forming units counted after 10 days (mean ± SD, $n = 7$; $P = 0.016$ and $P = 0.4$ for IC_{50} ($p53^{+/+}$ vs. $p53^{-/-}$) in *c* and *d*, respectively).

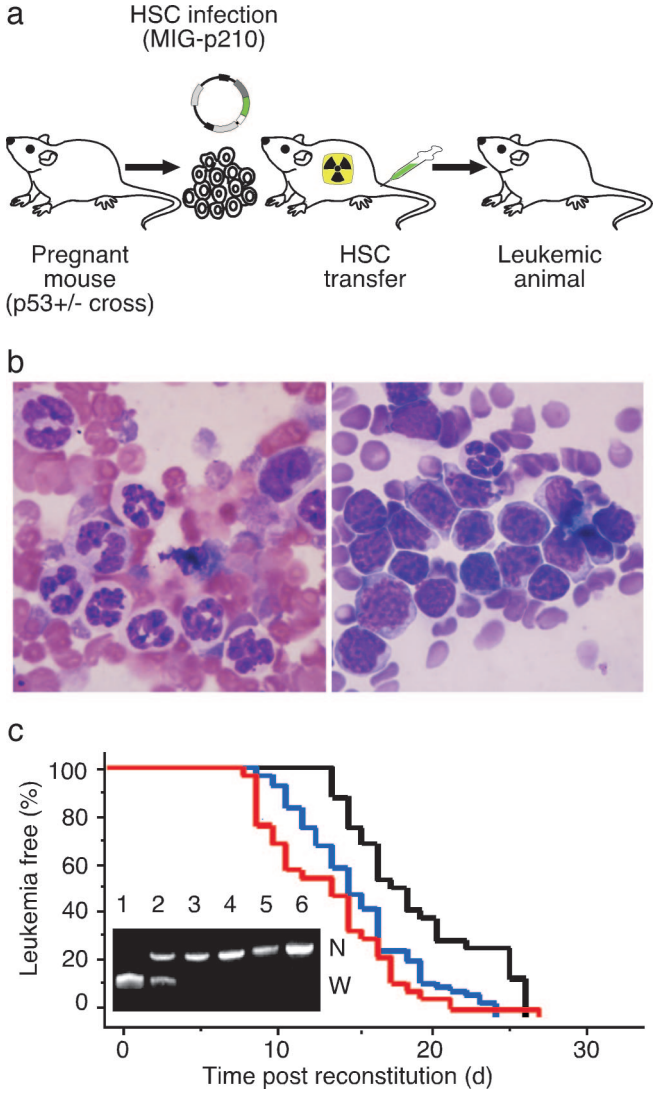

Fig. 3. BCR-ABL induced leukemia *in vivo*. (*a*) Schematic of the generation of mice harboring leukemias of defined *p53* status; MIG-p210: MSCV-p210-IRES-GFP. (*b*) Representative microphotographs of blood smears illustrating the resulting pathologies. Most animals develop a CML-like myeloproliferative disease (*Left*), whereas some have acute leukemias (*Right*). (*c*) Latency to leukemia onset after transplantation (day 0) of *BCR-ABL*-transduced HSCs of these genotypes: $p53^{+/+}$ (black, $n = 28$), $p53^{+/-}$ (blue, $n = 57$), and $p53^{-/-}$ (red, $n = 30$); $P = 0.0008$ ($p53^{+/+}$ vs. $p53^{+/-}$); $P = 0.0005$ ($p53^{+/+}$ vs. $p53^{-/-}$); $P = 0.18$ ($p53^{+/-}$ vs. $p53^{-/-}$). (*Inset*) PCR to detect loss of heterozygosity in the *p53* locus. N, the knockout allele; W, the wild-type allele. Lane 1, $p53^{+/+}$ control; lane 2, $p53^{+/-}$ control; lane 3–6, CML samples derived from $p53^{+/-}$ HSCs.

To test the impact of imatinib and *p53* on the proliferative capacity of these BCR-ABL-expressing hematopoietic progenitors, we performed colony assays in methylcellulose. Here, cells derived from $p53^{+/+}$ and $p53^{-/-}$ fetal livers were transduced with a BCR-ABL retrovirus that coexpresses GFP such that 15–30% of the population was infected and, thus, expressed both BCR-ABL and GFP. Non-GFP-expressing cells served as an internal control to assess the contribution of BCR-ABL to drug action. Cells were plated in methylcellulose in the presence of various concentrations of imatinib, and colonies were quantified and inspected for GFP fluorescence 10 days later. Consistent with a role for *p53* in suppressing BCR-ABL transformation (18), $p53^{-/-}$ cells produced ≈10-fold more BCR-ABL-expressing colonies than the $p53^{+/+}$ controls (data not shown). As expected, imatinib potently suppressed colony formation by $p53^{+/+}$ HSCs expressing BCR-ABL with an IC_{50} of ≈0.05 μM. By contrast, $p53^{-/-}$ HSC-expressing BCR-ABL were less sensitive to BCR-ABL inhibition, displaying an increase in IC_{50} ($IC_{50} = 2.1$ μM) (Fig. 2 *b* and *c*). Interestingly, colony formation by non-BCR-ABL-expressing cells was not substantially influenced by *p53* status (IC_{50} ($p53^{+/+}$) = 3.2 μM, IC_{50} ($p53^{-/-}$) = 3.8 μM) (Fig. 2*d*). Therefore, *p53* can modulate the cellular effects of oncogene inhibition and contribute to the therapeutic index of imatinib therapy.

***p53* Loss Reduces Leukemic Cell Clearance and Survival in Mice Harboring BCR-ABL Leukemias.** We generated mice bearing leukemias of defined *p53* status by infecting HSCs derived from $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ fetal livers with a BCR-ABL/GFP retrovirus transferred into lethally irradiated recipients (Fig. 3*a*). Consistent with previous reports (27), these animals developed a CML-like myeloproliferative disease and, occasionally, an acute leukemia. Although *p53* loss did not impact the range of pathologies we observed, the onset of leukemias in mice reconstituted with $p53^{-/-}$ cells was more rapid than controls, and leukemias arising from $p53^{+/-}$ cells invariably lost the wild-type *p53* allele (Fig. 3 *b*, *c*, and *Inset*). Still, BCR-ABL-expressing leukemias arising in $p53^{+/+}$ cells retained an intact *p53* pathway, because p53 levels and activity (as measured by expression of its transcriptional target p21) were increased after treatment of leukemia-bearing mice with doxorubicin, a conventional chemotherapeutic agent known to activate *p53* (see Fig. 8, which is

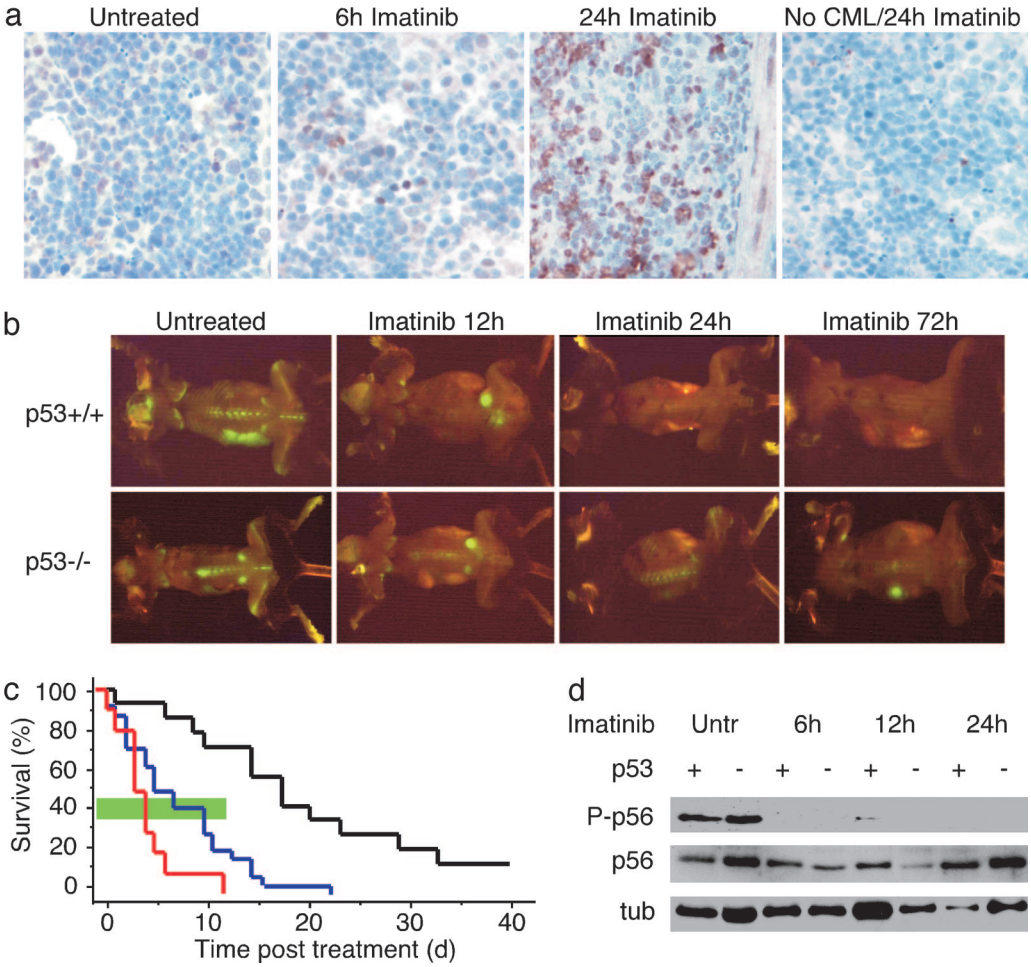

Fig. 4. *p53* and targeted therapy in murine CML. (*a*) Representative immunohistochemical stains to assess p53 expression in the spleens of healthy vs. leukemic mice treated as indicated. (*b*) Fluorescence imaging of a cohort of $p53^{+/+}$ and $p53^{-/-}$ leukemia-bearing mice killed at various times after imatinib treatment. Representative examples are shown. (*c*) Kaplan–Meier plot detailing survival times of leukemic mice grouped by genotype upon imatinib treatment; imatinib was started at the onset of leukemia (day 0), and a green bar indicates the treatment interval. Leukemias are derived from *BCR-ABL*-transduced $p53^{+/+}$ HSCs (black, $n = 15$), $p53^{+/-}$ HSCs (blue, $n = 24$), or $p53^{-/-}$ (red, $n = 10$); $P = 0.0002$ ($p53^{+/+}$ vs. $p53^{+/-}$); $P < 0.0001$ ($p53^{+/+}$ vs. $p53^{-/-}$); $P = 0.08$ ($p53^{+/-}$ vs. $p53^{-/-}$). (*d*) Bone marrow lysates of $p53^{+/+}$ (+) or $p53^{-/-}$ (−) leukemias were prepared from untreated animals (Untr) or at various times after a single treatment with imatinib and subjected to immunoblotting with antibodies against phosphorylated and total p56 (P-p56, p56) and tubulin (tub).

published as supporting information on the PNAS web site). Thus, as occurs in other mouse models of BCR-ABL-induced leukemia (28) and in human CML, *p53* loss conferred an advantage to BCR-ABL-expressing cells during leukemogenesis.

To test the impact of *p53* on imatinib responses *in vivo*, animals were treated upon leukemia manifestation with a 2-week twice-daily schedule of 100 mg/kg of body weight imatinib (29). One cohort of mice was killed at various times after treatment to assess p53 induction and BCR-ABL kinase inhibition, whereas others were monitored for treatment responses by fluorescence imaging or peripheral blood analysis. Consistent with *in vitro* results, we found an induction of p53 in spleens of leukemic animals but not in normal, nonleukemic mice (Fig. 4*a*; Fig. 9*a*, which is published as supporting information on the PNAS web site; and data not shown). Furthermore, whereas $p53^{+/+}$ leukemias showed substantial apoptosis in the peripheral blood and were completely cleared, *p53* mutant leukemias underwent less apoptosis and only partial responses to imatinib therapy, as determined by blood counts and fluorescence imaging (Figs. 4*b* and 9*b*).

A cumulative analysis of the treatment data for survival (Fig. 4*c*) and time to relapse (see Fig. 10, which is published as supporting information on the PNAS web site) confirmed a modifying effect of *p53* on imatinib response. Although all animals bearing *p53*-deficient leukemias, generated from either $p53^{-/-}$ or $p53^{+/-}$ HSCs, succumbed to their disease by day 25 after the start of imatinib therapy; nearly 40% of animals with $p53^{+/+}$ leukemias lived beyond this point (Fig. 4*c*). Although imatinib clearly has *p53*-independent activities, these results demonstrate that *p53* loss can impede the response to imatinib therapy *in vivo*.

Imatinib Efficiently Inhibits BCR-ABL Kinase Activity in p53-Deficient Cells. *p53* mutations produce drastic changes in cell physiology and can promote genomic instability (30, 31). Although it is possible that such secondary changes contribute to the decreased imatinib sensitivity we observe in *p53*-deficient leukemias, our *in vitro* data imply that *p53* can directly influence cellular responses to imatinib (see Figs. 1 and 2). To determine whether *p53* loss impairs imatinib action by preventing effective BCR-ABL kinase inhibition (as occurs in cells harboring p210/T315I) or through a downstream mechanism, we monitored BCR-ABL kinase activity in leukemic bone marrow isolates from imatinib-treated mice. Here, phosphorylation of $p56^{Dok\text{-}2}$ (a surrogate marker for kinase activity) was repressed within 6 h after a single dose of imatinib, and this repression was sustained for up to 24 h. More

importantly, *p53*-expressing and -deficient leukemias showed similar reductions in $p56^{Dok\text{-}2}$ phosphorylation after imatinib treatment (Fig. 4*D*). Therefore, *p53* loss does not interfere with the ability of imatinib to inhibit BCR-ABL kinase activity but, instead, attenuates the cellular response to BCR-ABL inhibition.

***p53* Mutations Can Accompany Progression to Imatinib Resistance in Some CML Patients.** Our studies use several well characterized models of *BCR-ABL* transformation to establish that parallel pathways independent from those emanating from the primary oncogenic signal can influence the response to targeted cancer therapy. Although our focus on BCR-ABL and imatinib reflected our desire to study a well characterized model of targeted therapy, our results raise the possibility that these observations may be relevant to human CML. We therefore surveyed a heterogeneous set of 50 specimens from CML patients, most of whom started imatinib in late disease stages and developed hematologic resistance to the drug (8). Ten resistant samples (20%) had evidence of cytogenetic alterations on chromosome 17, where *p53* resides, and 29 (58%) had point mutations in the BCR-ABL kinase domain. Only two resistant specimens showed both *BCR-ABL* mutations and chromosome 17 abnormalities. However, of the resistant cases that displayed chromosome 17 alterations without *BCR-ABL* mutations, three of five cases examined by DNA sequence analysis showed *p53* mutations that were not present in the preimatinib samples (Table 1, which is published as supporting information on the PNAS web site). Thus, *p53* mutations can accompany progression to imatinib resistance in human CML.

Discussion

We show that disruption of *p53* in *BCR-ABL*-transformed cells can impede sensitivity to imatinib. In contrast to the complete resistance conferred by mutations in the BCR-ABL kinase, *p53* loss does not prevent BCR-ABL kinase inhibition but attenuates its antiproliferative effects and modifies imatinib responses *in vivo*. As a consequence, *p53* loss impedes the clearance of leukemic cells, which may increase the population of surviving cells prone to additional progression- and resistance-promoting mutations. In this manner, *p53* mutations, or other mutations affecting the *p53* network, may contribute to the increase in refractory cases in blast crisis CML or in Ph^+ (Philadelphia chromosome) acute lymphoblastic leukemia. Such a possibility is consistent with our limited analysis of matched patient samples and clinical data linking *p53* mutations to blast crisis and poor outcome and linking an apoptosis-defective *p53* variant (Pro-72) to imatinib failure in patients (15, 32).

Studies in cultured cell lines have noted variable effects of BCR-ABL inhibition on p53 levels and activity (19–22). Using BCR-ABL-transformed BaF/3 cells, primary fetal liver HSCs, and a murine model of CML, we see that, in all cases, p53 is induced in response to imatinib treatment, and, at least *in vitro*, this finding correlates with a decreased phosphorylation of Akt and Mdm2. Other mechanisms may also contribute to p53 activation after imatinib treatment, including effects of BCR-ABL on Mdm2 translation and checkpoint pathways involved in DNA damage responses (19, 33). Paradoxically, whereas BCR-ABL kinase inhibition can induce p53 in transformed cells, v-Abl or BCR-ABL expression can induce p53 via the ARF pathway to restrain transformation (ref. 18; data not shown). Presumably, BCR-ABL simultaneously induces both pro- and antisurvival signals that impact p53 regulation such that acute ablation of BCR-ABL kinase activity has a greater impact on those signals that restrain p53 action, revealing their antiproliferative potential. Other parallel signaling networks may also impact how a cell responds to BCR-ABL inhibition, thus producing heterogeneity in treatment responses.

The treatment of BCR-ABL-transformed cells with imatinib represents the paradigm of targeted cancer therapy. Irrespective of the overall impact of *p53* mutations on response to imatinib therapy in CML patients, our results have implications for the use of targeted therapies in the clinic. For example, we demonstrate that *p53* contributes to the dependence of cancer cells on the continued activity of an initiating oncogene, sometimes called oncogene addiction (2), such that *p53* loss attenuates the cellular response to oncogene inactivation. These findings suggest that *p53* might contribute to the antitumor activity of other targeted therapeutics, and it is noteworthy that *p53* loss facilitates oncogene-independence and the eventual outgrowth of tumors in conditional transgenic mice upon inactivation of the *wnt* (34) oncogene. Our results also illustrate how mutations that accompany disease progression can reduce the efficacy of targeted therapy, presumably because drug action relies, in part, on genes and processes that normally limit disease progression (e.g., *p53*). In this view, the response of tumor cells to targeted therapy displays some parallels to conventional therapy, which can also depend on *p53* or other stress-response networks for best efficacy. Together, these results may help explain the heterogeneous response of tumors to targeted therapeutics and provide clues for their optimal use.

Materials and Methods

Cell Lines and Gene Transfer. Ba/F3 cells, 32D cells, and primary HSCs were stably transduced with MSCV-IRES-GFP vectors expressing BCR-ABL or an imatinib-resistant and kinase-active T315I mutant (a generous gift from C. L. Sawyers, University of California, Los Angeles). Ba/F3/p210, Ba/F3/T315I, or 32D cells expressing BCR-ABL were also transduced with two RNAi vectors against *p53* [shp53C (25) shp53D/1224 (26)] or an MSCV control vector. Retroviral gene transfer was performed as described in ref. 35.

Western Blot Analysis. Immunoblots were performed from whole-cell lysates (36). Antibodies against α-tubulin (1:5,000, B-5–1-2; Sigma), p56 and phosphorylated p56 (1:1,000, Cat#s 3914 and 3911; Cell Signaling Technology, Beverly, MA), p53 (1:500, p53–505; NovoCastra, Newcastle upon Tyne, U.K.), p21 (C19, SC-397; Santa Cruz Biotechnology), phosphorylated (Ser-473) Akt (1:1,000, Cat# 4051; Cell Signaling Technology), total Akt (1:1000, Cat# 2966; Cell Signaling Technology), total Mdm2 (clones 2A10 and 4B11 each at 1:50, a gift from A. Levine, Institute for Advanced Study, Princeton) (37), and phosphorylated (ser166) Mdm2 (1:1,000, Cat# 3521; Cell Signaling Technology) were used as probes and detected by using enhanced chemiluminescence.

***In Vitro* Drug-Response Assays.** For "competition" assays, Ba/F3/p210 or Ba/F3/T315I cells were partially transduced with an RNAi vector (p53C or p53D) or a control vector and propagated for 1 week in the presence of absence of 1 μM imatinib in standard media. The percentage of GFP-expressing cells was determined by flow cytometry. For methylcellulose assays, fetal liver $p53^{+/+}$ and $p53^{-/-}$ HSCs (embryonic day 13–15) were derived and retrovirally transduced as described in ref. 35. Viability was determined by Trypan blue exclusion, and 1×10^3 viable cells per well were plated in Methocult GF media (Cat# 3534; StemCell Technologies, Vancouver) in the presence or absence of imatininb. The total number and the number of BCR-ABL/GFP-expressing colony-forming units were determined 10 days after plating.

Generation of Mice. The murine model of a retrovirally induced CML-like disease has been described in ref. 27. We modified the model using fetal liver HSCs (embryonic day 13–15) from a cross of $p53^{+/-}$ C57BL/6 mice. Determination of *p53* status was by

allele-specific PCR (12). After reconstitution, the mice were monitored by blood counts. Leukemic mice (counts >50,000 per μl) were treated or bone marrow and spleen were harvested for pathology or *in vitro* studies. Preparation and staining of tissue samples and cytospin preparations were according to published recommendations (38). Mice of both $p53^{+/+}$ and $p53^{-/-}$ genotypes were later diagnosed with a CML-like myeloproliferative disease in two of three cases and an acute (mostly lymphocytic) leukemia in one of three cases.

Treatment Studies. Leukemic mice were treated with 100 mg/kg of body weight imatinib twice daily i.p. over 2 weeks. Mice were monitored for response by blood counts. A complete remission was defined as absence of leukemia, and leukemia-free survival was defined as the duration of a complete remission between treatment and relapse. Whole-body fluorescence imaging was performed as described in ref. 12. Disease onset and treatment data were analyzed in Kaplan–Meier format and logrank (Mantel–Cox) for statistical significance. IC_{50} data from separate and duplicate experiments ($n = 7$) were compared by using a t test and are shown as mean ± SD.

***p53* Sequencing.** Patient selection, response criteria, cytogenetics, and analysis of *BCR-ABL* were as described in ref. 8. Generation of cDNAs and sequencing of the *p53* sequences corresponding to *p53* exons 2–11 were performed by using published primers and an established protocol (39).

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1. Sawyers, C. (2004) *Nature* **432,** 294–297.
2. Weinstein, I. B. (2002) *Science* **297,** 63–64.
3. Jonkers, J. & Berns, A. (2004) *Cancer Cell* **6,** 535–538.
4. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N. & Sawyers, C. L. (2001) *Science* **293,** 876–880.
5. Shah, N. P., Tran, C., Lee, F. Y., Chen, P., Norris, D. & Sawyers, C. L. (2004) *Science* **305,** 399–401.
6. Tipping, A. J., Baluch, S., Barnes, D. J., Veach, D. R., Clarkson, B. M., Bornmann, W. G., Mahon, F. X., Goldman, J. M. & Melo, J. V. (2004) *Leukemia* **18,** 1352–1356.
7. Druker, B. J., Sawyers, C. L., Kantarjian, H., Resta, D. J., Reese, S. F., Ford, J. M., Capdeville, R. & Talpaz, M. (2001) *N. Engl. J. Med.* **344,** 1038–1042.
8. Lahaye, T., Riehm, B., Berger, U., Paschka, P., Muller, M. C., Kreil, S., Merx, K., Schwindel, U., Schoch, C., Hehlmann, R. & Hochhaus, A. (2005) *Cancer* **103,** 1659–1669.
9. Lange, T., Park, B., Willis, S. G. & Deininger, M. W. (2005) *Cell Cycle* **4,** 1761–1766.
10. Lowe, S. W., Cepero, E. & Evan, G. (2004) *Nature* **432,** 307–315.
11. Harris, S. L. & Levine, A. J. (2005) *Oncogene* **24,** 2899–2908.
12. Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M. & Lowe, S. W. (2002) *Cancer Cell* **1,** 289–298.
13. Ahuja, H., Bar-Eli, M., Advani, S. H., Benchimol, S. & Cline, M. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6783–6787.
14. Kelman, Z., Prokocimer, M., Peller, S., Kahn, Y., Rechavi, G., Manor, Y., Cohen, A. & Rotter, V. (1989) *Blood* **74,** 2318–2324.
15. Nakai, H. & Misawa, S. (1995) *Leuk. Lymphoma* **19,** 213–221.
16. Mashal, R., Shtalrid, M., Talpaz, M., Kantarjian, H., Smith, L., Beran, M., Cork, A., Trujillo, J., Gutterman, J. & Deisseroth, A. (1990) *Blood* **75,** 180–189.
17. Feinstein, E., Cimino, G., Gale, R. P., Alimena, G., Berthier, R., Kishi, K., Goldman, J., Zaccaria, A., Berrebi, A. & Canaani, E. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6293–6297.
18. Thome, K. C., Radfar, A. & Rosenberg, N. (1997) *J. Virol.* **71,** 8149–8156.
19. Trotta, R., Vignudelli, T., Candini, O., Intine, R. V., Pecorari, L., Guerzoni, C., Santilli, G., Byrom, M. W., Goldoni, S., Ford, L. P., *et al.* (2003) *Cancer Cell* **3,** 145–160.
20. Goetz, A. W., van der Kuip, H., Maya, R., Oren, M. & Aulitzky, W. E. (2001) *Cancer Res.* **61,** 7635–7641.
21. Brusa, G., Mancini, M., Campanini, F., Calabro, A., Zuffa, E., Barbieri, E. & Santucci, M. A. (2005) *Acta Haematol.* **114,** 150–154.
22. Goldberg, Z., Levav, Y., Krichevsky, S., Fibach, E. & Haupt, Y. (2004) *Cell Cycle* **3,** 1188–1195.
23. Skorski, T., Bellacosa, A., Nieborowska-Skorska, M., Majewski, M., Martinez, R., Choi, J. K., Trotta, R., Wlodarski, P., Perrotti, D., Chan, T. O., *et al.* (1997) *EMBO J.* **16,** 6151–6161.
24. Mayo, L. D. & Donner, D. B. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11598–11603.
25. Hemann, M. T., Fridman, J. S., Zilfou, J. T., Hernando, E., Paddison, P. J., Cordon-Cardo, C., Hannon, G. J. & Lowe, S. W. (2003) *Nat. Genet.* **33,** 396–400.
26. Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Hannon, G. J. & Lowe, S. W. (2005) *Nat. Genet.* **37,** 1289–1295.
27. Pear, W. S., Miller, J. P., Xu, L., Pui, J. C., Soffer, B., Quackenbush, R. C., Pendergast, A. M., Bronson, R., Aster, J. C., Scott, M. L. & Baltimore, D. (1998) *Blood* **92,** 3780–3792.
28. Honda, H., Ushijima, T., Wakazono, K., Oda, H., Tanaka, Y., Aizawa, S., Ishikawa, T., Yazaki, Y. & Hirai, H. (2000) *Blood* **95,** 1144–1150.
29. Wolff, N. C. & Ilaria, R. L., Jr. (2001) *Blood* **98,** 2808–2816.
30. Hartwell, L. H. & Kastan, M. B. (1994) *Science* **266,** 1821–1828.
31. Brusa, G., Benvenuti, M., Mazzacurati, L., Mancini, M., Pattacini, L., Martinelli, G., Barbieri, E., Greenberger, J. S., Baccarani, M. & Santucci, M. A. (2003) *Haematologica* **88,** 622–630.
32. Bergamaschi, G., Merante, S., Orlandi, E., Galli, A., Bernasconi, P. & Cazzola, M. (2004) *Haematologica* **89,** 868–869.
33. Deng, X., Hofmann, E. R., Villanueva, A., Hobert, O., Capodieci, P., Veach, D. R., Yin, X., Campodonico, L., Glekas, A., Cordon-Cardo, C., *et al.* (2004) *Nat. Genet.* **36,** 906–912.
34. Gunther, E. J., Moody, S. E., Belka, G. K., Hahn, K. T., Innocent, N., Dugan, K. D., Cardiff, R. D. & Chodosh, L. A. (2003) *Genes Dev.* **17,** 488–501.
35. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. (1997) *Cell* **88,** 593–602.
36. Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., Kogan, S., Cordon-Cardo, C., Pelletier, J. & Lowe, S. W. (2004) *Nature* **428,** 332–337.
37. Chen, J., Marechal, V. & Levine, A. J. (1993) *Mol. Cell. Biol.* **13,** 4107–4114.
38. Kogan, S. C., Ward, J. M., Anver, M. R., Berman, J. J., Brayton, C., Cardiff, R. D., Carter, J. S., de Coronado, S., Downing, J. R., Fredrickson, T. N., *et al.* (2002) *Blood* **100,** 238–245.
39. Thirion, A., Rouanet, P., Thezenas, S., Detournay, D., Grenier, J. & Lopez-Crapez, E. (2002) *Oncol. Rep.* **9,** 1167–1172.

Legends for supplementary figures (supporting online material)

Supplementary Figure 1. p53 stabilization by BCR-ABL inhibitors. Immunoblot analysis of lysates prepared from Ba/F3 cells stably transduced with empty vector, BCR-ABL (p210) or the T315I mutant BCR-ABL (p210/T315I) treated with PD166326 as indicated and probed for p53, phosphorylated and total levels of p56 (P-p56 and p56, respectively).

Supplementary Figure 2. *In vitro* competition assay. Populations of Ba/F3 cells stably expressing either BCR-ABL (p210; **a and c)** or the T315I mutant (p210/T315I; **b)** were partially transduced with an RNAi vector targeting p53 (p53C) (**a** and **b**) or a control vector (MSCV-GFP) (**c**) and propagated in the presence or absence of 1μM imatinib for 1 week and then subjected to flow cytometry to determine the fraction of cells containing the RNAi vector (high GFP expression).

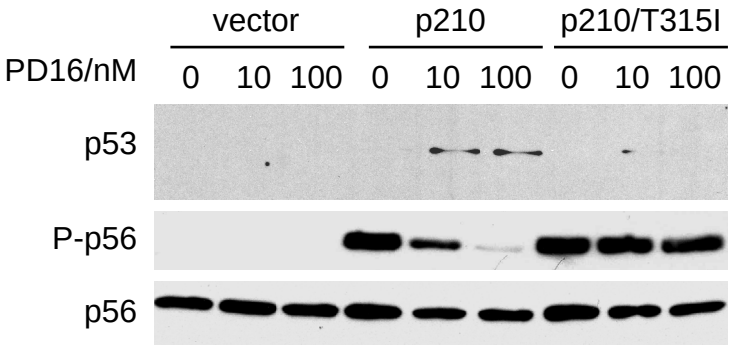
Supplementary Figure 3. *In vitro* competition assay with 32D cells. Populations of 32D cells stably expressing BCR-ABL were either transduced with an RNAi vector targeting p53 (p53C; empty squares) or a control vector (MSCV-GFP; filled circles) and incubated in the presence or absence of imatinib at the indicated concentrations for 1 week and then subjected to flow cytometry to determine the fraction of cells containing the RNAi or control vector (high GFP expression).

Supplementary Figure 4 Leukemias derived from p53+/+ HSCs retain an intact *p53* response. Immunoblot for p53, p21 and tubulin (tub) on lysates prepared from bone marrow of mice bearing *p53*+/+ or *p53*-/- leukemias untreated or harvested 6 hours following *in vivo* doxorubicin treatment (10 mg/kg i.p.).

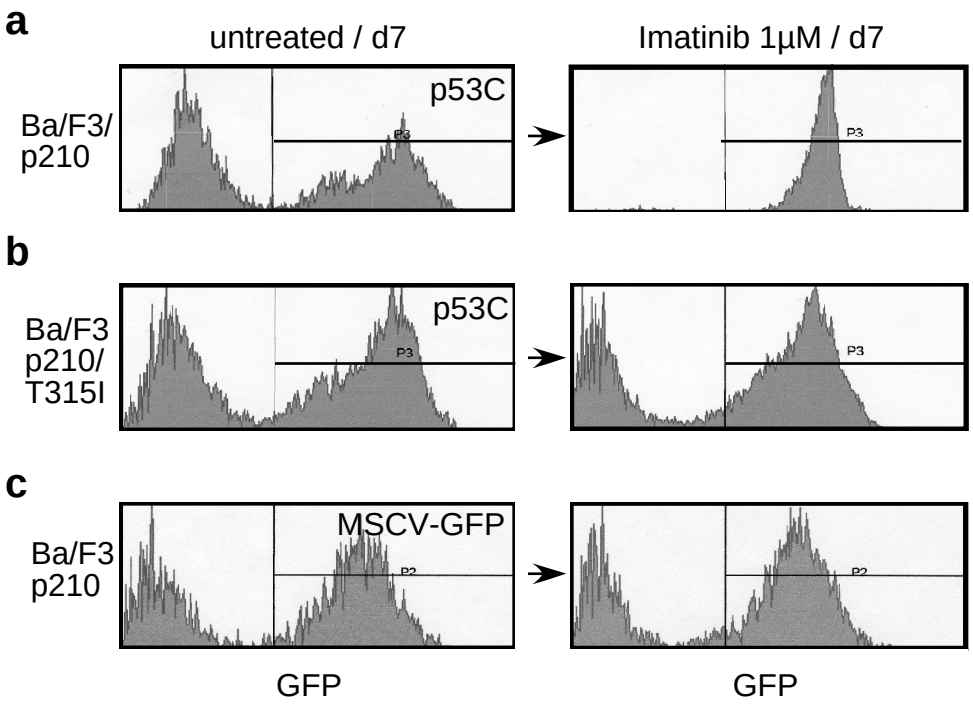
Supplementary Figure 5 Imatinib stabilizes p53 and induces apoptosis *in vivo*. (**a**) Immunoblot of lysates prepared from the spleens of leukemic mice harvested without treatment or 6 and 24 hours after therapy and probed for p53, GFP and tubulin (tub) as loading control. (**b**) Representative UV-fluorescence microphotographs of TUNEL-stained blood slides collected from mice bearing *p53*+/+ or *p53*-/- leukemias at the indicated times following imatinib administration.

Supplementary Figure 6 *In vivo* effects of imatinib treatment. Kaplan-Meier plot detailing leukemia-free survival of mice bearing *p53*+/+ (black, n=15) or *p53*-/- (red, n=34) leukemias following treatment with imatinib ($p<.0001$). A green bar indicates the treatment interval. Leukemias derived from *p53*+/- HSCs underwent *p53* LOH and showed no significant difference from leukemias derived from *p53*-/- HSCs and were considered jointly as *p53*-/- leukemias.

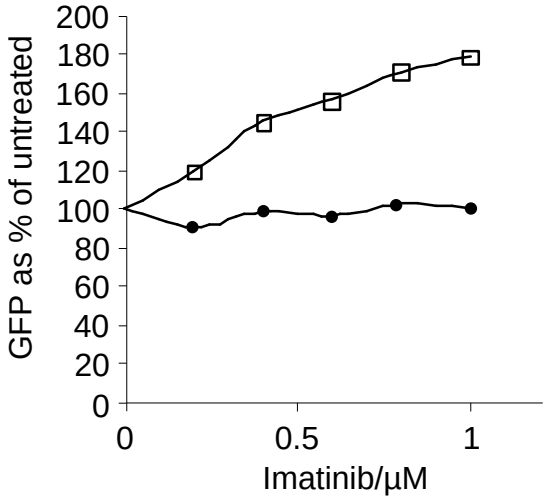
Supplementary Figure 1



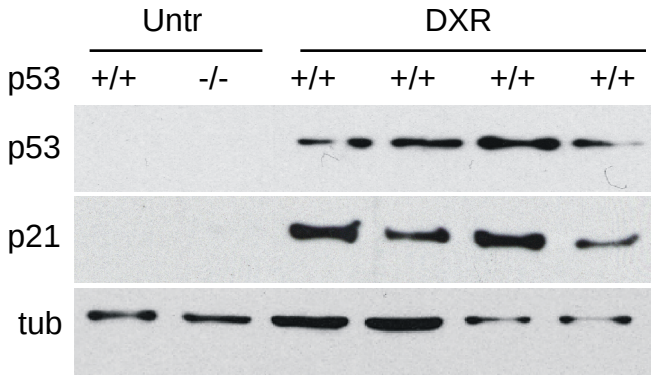
Supplementary Figure 2



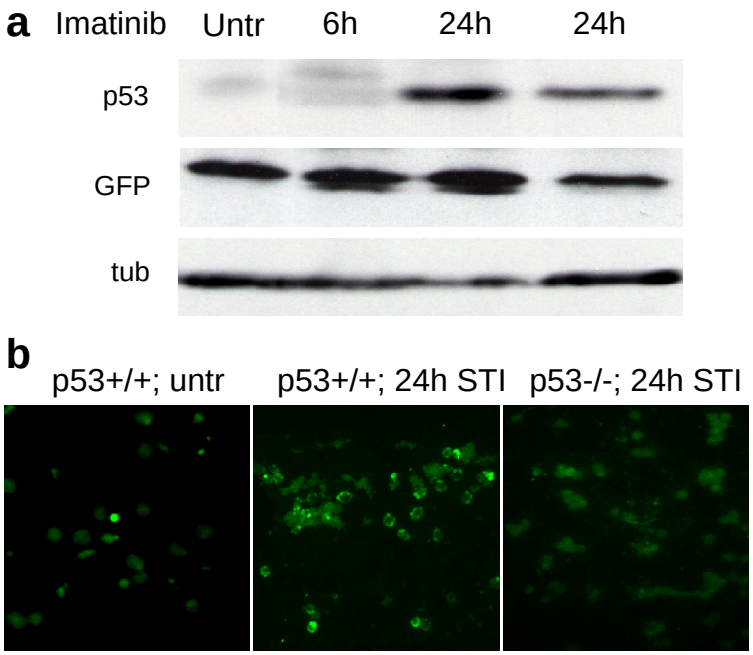
Supplementary Figure 3



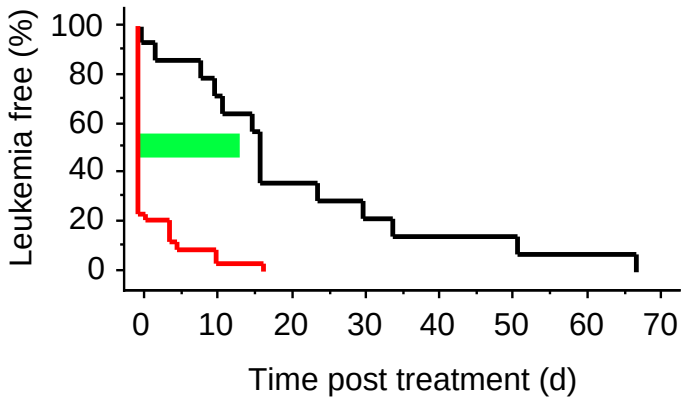
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Suppl. Table 1: Cytogenetics, *p53* and *BCR-ABL* mutational status before and after imatinib resistance in matched patient samples. Stage at start of imatinib therapy is shown: BC: blast crisis; AP: accelerated phase; CP: chronic phase.

Sample	**Stage**	**Before/after imatinib**	**Cytogenetics**	**Iso(17)**	***p53* mutation**	***BCR-ABL* mutation**
Pat. A	BC	Before	dup(2)(q31q36) [23/25]	N	N	N
	BC	After	dup(2) [26/26], **i(17)(q10)** [8/26]	Y	N	N
Pat. B	BC	Before	der(1)t(1;9;22)(qter>p22::q22>q34::q11>q13),der(7)(q?), der(9)t(9;1)(q22p22), del(11)(q23), -16, **del(17)(p11),** der(17)t(17;?)(p11>q25::?), add(19)(q13)	-	N	N
	BC	After	n.a.	-	**Y**	N
Pat. C	BC	Before	None	N	N	N
	BC	After	**iso(17q)** [23/26], +8 [1/26]	Y	N	N
Pat. D	AP	Before	**-17**[18/25], ins(7;17) [1/25]	-	N	N
	AP	After	**-17**[20/20], 6p+/17p-/19q+[10/20]	-	**Y**	N
Pat. E	CP	Before	add(9)(q34) [16/20]	N	N	N
	CP	After	+8, der(9)t(9;16)(q34;p11), -16, **i(17)(q10),** +der(22)t(9;22)(q34;q11) [17/25]	Y	**Y***	N

(*: relapse sample shows loss of the more apoptosis competent Arg72 allele)